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ENVIRONMENTAL ASPECTS OF THE DEGRADATION POTENTIAL OF ANAEROBIC BACTERIA

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SUMMARY

A short survey is presented on anaerobic transformation of hydrocarbons, ether compounds, and aromatics which, in the presence of oxygen, are primarily attacked by oxygenase reactions. Recent studies revealed that the biodegradative capacity of anaerobic microbial communities is by far higher than usually assumed. Several new types of anaerobic cleavage and elimination reactions were discovered in the recent past and open the view to a broad and fascinating variety of new catabolic pathways in anaerobic microbial communities. Implications of these degradative activities on microbial transformation of natural and synthetic organic compounds are discussed with respect to their energetics, the recalcitrance of pollutants in natural environments, and application of anaerobic systems for waste treatment processes.

INTRUDUCTION

The photosynthetic activity of green plants and cyanobacteria (about $1.3 \cdot 10^{17}$ g CO_2 per year; ref. 1) accumulated during earth's history enormous amounts of molecular oxygen in the atmosphere which allows today the lion's share of biotic production to be remineralized aerobically to H_2O and CO_2. Anaerobic degradation processes are only of minor importance which, however, cannot be measured exactly. Wherever organic matter accumulates to a major extent, aerobic degradation consumes oxygen and subsequently leads to complete anoxia if oxygen supply by diffusion processes is impaired. Thus, anaerobic niches are created by aerobic and facultatively anaerobic bacteria (ref. 2) and can be maintained at sizes as small as a few bacterial cell layers, e.g. in a bacterial colony (ref. 3) or as large as the hypolimnion of the Black Sea or the Gulf of Mexico. Anaerobes in such niches degrade a given substrate incompletely and provide the surrounding aerobes with reduced products which in turn are oxidized aerobically. Among those reduced intermediates are the classical fermentation products, but also the products of anaerobic respiration processes, e.g. ammonia, hydrogen sulfide, and methane. The amount of biogenic methane reaching the earth's atmosphere ($1.01 \cdot 10^{14}$ g CH_4 per year; ref. 4) is small compared to photosynthetic production, and makes up only about less than 0.1% of the latter. However, this rate only represents the small fraction of methane that reaches the atmosphere, and does not include all that methane which has already been reoxidized during its passage from the anoxic to the oxic world. With microbially produced hydrogen sulfide, the situation is even worse since in this case also chemical oxidation processes compete with microbial sulfide oxidation (ref. 5). From these considerations it appears that estimates on the global importance of anaerobic degradation activities are hard to make and will always imply extreme uncertainties.

The still increasing pollution of surface waters converted the sediments of rivers and lakes as well as hypolimnetic parts of stagnant water bodies into oxygen-deprived environments which often undergo complete anaerobiosis. Anaerobic sewage digesters are an example of man-made anaerobic environments in which increasing amounts of organic compounds are being decomposed in the absence of oxygen. Last not least, the gastrointestinal tract of higher animals including man represents another anoxic environment in which a broad variety of natural and synthetic compounds undergoes modification and degradation without the participation of molecular oxygen. Realizing this one can no longer maintain the idea that anaerobic degradative processes are only of marginal interest to the environmentally oriented microbiologist.

After consumption of oxygen in an oxygen-limited environment, nitrate and afterwards sulfate can take over its role as a terminal acceptor for the electrons released during substrate oxidation. Finally, carbon dioxide is reduced to methane which normally escapes back into the oxygenic world. Thus, oxygen's function as an electron acceptor can be taken over by other oxidized inorganic compounds. However, oxygen also serves an important function as reaction partner in the primary attack on certain substrates such as hydrocarbons, ether compounds, or aromatic compounds by means of mono- and dioxygenase reactions. Due to the unique chemistry of oxygen (refs. 6,7), its role in oxygenase reactions cannot be taken over by any other oxidized compound under anaerobic conditions. Therefore, degradation processes which in the presence of oxygen involve

oxygenase reactions have to proceed in its absence in an entirely different manner, if degradation is possible at all. It is only of minor importance then if nitrate, sulfate, or carbon dioxide are the terminal electron acceptors. It is not astonishing from the above considerations that the biodegradative potential of anaerobic bacteria was usually considered to be inferior to that of aerobes.

The search for limits of the biodegradative potential of anaerobes has two basic aspects: On the one hand, lack of degradation of certain compounds will lead to their accumulation in anoxic environments since many metabolic ways may lead to them but no one any further. On the other hand, proof of anaerobic biodegradability for a certain compound or whole groups of compounds provides the chance to use anaerobic processes for degradation of certain waste materials which, for whatever reason, are only insufficiently treated by the usual aerobic techniques. Thus, exploration of the anaerobic biodegradative potential implies both ecological and technological aspects, and both together can contribute to a better understanding of environmental problems and to their solution.

The present contribution wants to give a short survey on some recently discovered types of anaerobic degradation reactions. A second part will deal with the energetical implications of anaerobic degradation processes on mixed microbial communities in food chains.

A more detailed survey on limits and principles of anaerobic degradation as well as its application in technical systems will appear soon in a handbook article (ref. 8) to which the reader is referred also concerning the quoted literature.

PART 1. NEW REACTIONS IN ANAEROBIC DEGRADATION OF ORGANIC MATTER

Oxygenases are typically involved in the initial oxygen-dependent attack on e.g. hydrocarbons, ether compounds, or aromatic substances. If such compounds can be degraded in the absence of molecular oxygen, degradation pathways different from the known ones have to be postulated. In the following contribution I want to present new results on anaerobic degradation of either group of compounds, and to show how these new informations contribute to a better understanding of anaerobic degradation processes.

Hydrocarbons

There is so far no convincing report in the literature on anaerobic degradation of a saturated aliphatic hydrocarbon molecule (refs. 8,9). This problem is of special interest with the most simple hydrocarbon compound, methane, since a sulfate-dependent methane oxidation could help to explain many so far difficult to understand distribution profiles of methane, sulfide, and sulfate in anoxic hypolimnia and sediments (refs. 10-13). So far, no bacterium is known which can catalyze a net methane oxidation in the absence of molecular oxygen although some contradicting reports exist in the literature. The proven conversion of $^{14}CH_4$ tracers to $^{14}CO_2$ in anoxic sediment samples (refs. 14,15) can be explained by the ability of methane bacteria to catalyze methane oxidation simultaneously with methane formation (refs. 15,16). Since methane oxidation in these bacteria makes up less than 0.3% of the simultaneous methane formation, this mechanism cannot explain a net methane oxidation as postulated. Thus sulfate-dependent methane oxidation remains an unsolved problem at present but it should still be considered as a challenge to microbial physiologists and ecologists.

Unlike saturated hydrocarbons, unsaturated ones are basically degradable in the absence of oxygen, and optimal degradation was observed with terminally unsaturated hydrocarbons. 1-Hexadecene was stoichiometrically converted to methane and CO_2 in mixed enrichment cultures (ref. 17). The initial step is probably a hydration to 1-hexadecanol which is further oxidized via palmitate and degraded by ß-oxidation. The hydration reaction is of special interest since it occurs in contradiction to Markownikoff's law: chemical hydration would lead to 2-hexadecanol, and its further oxidation would probably stop at the ketone stage. Squalene, a branched unsaturated isoprenoid hydrocarbon, was only incomplete degraded by methanogenic enrichment cultures; the amount of methane formed corresponded to about 50 % degradation (ref. 17). One could speculate that hydration and carboxylation reactions are primarily involved in this catabolic sequence. Uncontrolles reduction of double bonds could lead in part to saturated branched hydrocarbon derivatives which are hard to degrade any further.

Degradation of branched alkyl skeletons was studied in our laboratory with branched-chain fatty acids. The biochemical pathways used are basically similar to those employed by aerobic

bacteria, e.g. carboxylation of methyl branches (ref. 18; Stieb and Schink, manuscripts in preparation). However, special problems arise with the energy yields obtained. All syntrophic associations of anaerobes degrading branched-chain fatty acids grew extremely slowly, and the cell yields obtained were very small as well.

Benzenoid compounds appear to be anaerobically degradable if they carry at least one hydroxyl or one carboxyl function (refs. 8,19,20). Pathways for anaerobic degradation of benzene, toluene, xylene, ethylbenzene are hard to figure out, but anaerobic degradation of such compounds by hydroxylation reactions has been reported by several groups in the recent past (ref. 21; Grbic-Galic, Bossert et al., manuscripts in preparation). The biochemistry of the reactions involved in the primary attack on these compounds has still to be elucidated in detail.

Anaerobic and microaerobic degradation of hydrocarbon compounds bears some interesting aspects with respect to a possible application in the removal of underground oil spills. Recent publications report on successful degradation of hydrocarbons and other oil constituents in the apparent absence of oxygen in oil-contaminated areas (refs. 22,23). Although presence of oxygen traces in these experiments cannot be ruled out with certainty it appears evident that nitrate has a highly stimulating effect on hydrocarbon removal. Perhaps nitrate serves as electron acceptor in the terminal destruction of a primary oxidation product, and thus allows to save molecular oxygen for the primary oxygenase-dependent hydrocarbon activation. A simultaneous reduction of oxygen and nitrate with hydrocarbons as substrate was observed with a pure culture of Pseudomonas aeruginosa (ref. 24).

Ether linkages

Oxygen-dependent hydroxylases (monooxygenases) can transform an ether linkage into a half-acetal linkage by hydroxylation of one of the ether carbon atoms. A typical example is the demethoxylation of phenylmethyl ethers (ref. 25). Homoacetogenic bacteria can cleave off methoxyl groups from aromatic compounds in the absence of oxygen, and ferment them in the same manner as methanol to acetate (ref. 26). The chemistry of this phenyl ether cleavage reaction is still not clear; for several reasons, a direct transfer of the methyl residue to a B_{12}-carrier is assumed (ref. 8,27). Also dialiphatic ether linkages were shown to be

split in the absence of oxygen. High molecular weight polyethylene glycol is fermented to acetate and ethanol via intermediate acetaldehyde formation (refs. 28,29). Degradation starts from the free hydroxyl group at one end, and proceeds stepwise releasing C-2 moieties. Involvement of a B_{12}-enzyme which shifts the terminal hydroxyl group to the subterminal carbon atom appears to be most probable, however, has not yet been proven in cell-free extracts. The first degradation product identified was acetaldehyde, which would easily be released from an intermediately formed halfacetal compound (ref. 29).

Degradation of polyethylene glycol is of special interest because it represents the hydrophilic residue of nonionic surfactants (ref. 30). Aerobic degradation of these surfactants starts with cleavage of the polyethylene glycol chain from the lipophilic residue, and oxidizes both parts further separately (ref. 31). Anaerobic bacteria able to degrade polyethylene glycol can also attack nonionic surfactants, but they have to degrade the ethylene glycol chain stepwise beginning with the hydroxy terminal residue. Anaerobic degradation of these surfactants therefore includes uptake of the whole molecule into the cell, processing inside, and excretion of the lipophilic residue. These two transport processes limit considerably the variety of surfactant types which can be degraded anaerobically.

On the other hand, anaerobic degradation of these compounds appears to be more efficient than aerobic oxidation: Alkylphenylethoxylates are aerobically converted only to alkylphenylmono- or diethoxylate derivatives. During anaerobic treatment, these last ethoxylate residues are cleaved off, and the lipophilic alkylphenol is formed. These alkylphenols are not degraded any further and accumulate in anoxic sediments to highly toxic concentrations (ref. 32). This example shows that a high degradation capacity of a certain microbial community can also detrimentally affect an anaerobic environment by producing toxic derivatives from non-toxic precursors.

The case of polyethylene glycol allows some speculations on how a degradation pathways for a synthetic, non-natural compound evolved. Interestingly, the polyethylene glycol utilizing isolate Pelobacter venetianus grows with polymers down to the dimer but can use the monomer ethylene glycol for growth only in substrate-limited continuous culture (ref. 29). If ethylene glycol is provided at enhanced concentrations, the bacterium kills itself

by accumulation of high amounts (up to 10 mM) of acetaldehyde. Another bacterium isolated from the same environment, Pelobacter carbinolicus, resembles P. venetianus in many respects, however, grows well with the monomer ethylene glycol and cannot utilize dimers and higher polymers. If the above hypothesis is true that polyethylene glycol degradation proceeds via a coenzyme B_{12}-dependent shift of the terminal alcohol function to the subterminal carbon atom this reaction would be very similar to a classical diol dehydrase reaction (ref. 33). Thus, anaerobic polyethylene glycol utilization could have evolved from ethylene glycol fermentation by a structural or regulatory modification of the latter. The modified enyzme not only degrades polyethylene glycol but also the monomer, however, it is so active that it accumulates toxic concentrations of acetaldehyde if exposed to enhanced ethylene glycol concentrations. Further work in our lab will focus on the characterization of the polymer-degrading enzyme(s).

It can be concluded that ether linkages can be split in the absence of oxygen if a free alcohol group is available for a coenzyme B_{12}-dependent shift reaction. This is of importance also with respect to the stability of archaebacterial glycerol isopranoid ether lipids in anoxic sediments.

Aromatic compounds

Pathways for anaerobic degradation of benzoate derivatives have been suggested repeatedly in the recent past (refs. 19,20). Most authors agree in that the initial ring saturation occurs in one single step which transforms the benzenoid structure into a cyclohexane carboxylate derivative. Although detailed enzymatic studies are still lacking it is believed that this primary ring saturation reaction requires binding of benzoate to coenzyme A. The further ring opening reactions can be considered as a modified ß-oxidation pathway, and similar reactions may underlie the terminal oxidation of the ring skeleton. Problems arise if there is no carboxyl group available at the ring structure, e.g. with phenols. The pathways suggested for phenol degradation are not very satisfactory in this point and the experimental basis for new hypotheses is weak. Experiments in our own group in the recent past focused on the influence of hydroxyl substituents at the benzoate ring on its anaerobic biodegradability. Enrichment experiments were carried out with all isomers of mono-, di-,

and trihydroxybenzenes and sediment samples from various sources in the absence of nitrate or sulfate. The results obtained so far with a huge number of enrichment cultures and pure cultures can be summarized as follows:

1. Contrary to aerobic bacteria, anaerobic enrichment cultures proved to be highly specialized for the degradation of certain isomers. Already after 3 to 4 transfer with the same substrate, most enrichment cultures only utilized the respective isolation substrate and perhaps one or two structurally related compounds. None of the isolated pure or defined cultures degrading benzenoid compounds was able to grown with common substrates such as sugars or aliphatic organic acids and alcohols.
2. Degradation of some hydroxybenzoates (4-hydroxybenzoate, 3,4-dihydroxybenzoate, 2,4-dihydroxybenzoate, 1.3.5.- and 2.4.6.-trihydroxybenzoate) involved a primary decarboxylation leading to the respective mono-, di- and trivalent phenols. It is difficult to imagine how these phenols should be metabolized further via ring saturation because a carboxylic function, the prerequisite for coenzyme CoA-dependent saturation is lacking. Several alternative pathways of phenol degradation have been suggested for the various isomers which all imply a postulated destabilization of the ring structure by ketone-enol tautomerization (refs. 8, 34-37).
3. Hydroxylic groups in positions suited for a ß-oxidative ring cleavage reaction (e.g. with 2-hydroxybenzoate) appears to remain at the ring until it is cleaved. Contrary, hydroxylic groups in unsuited positions (e.g. with catechol, hydroquinone, 3-hydroxybenzoate) are first reductively cleaved off before ring degradation takes place. The dehydroxylated derivatives (phenol, benzoate) could be detected as degradation intermediates at considerable concentrations in the culture fluid (Refs. 34,37).
4. A reaction similar to reductive dehydroxylation was observed in enrichment cultures with anthranilic acid (2-aminobenzoate; ref. 35). This compound was transformed to benzoate which accumulated in the medium if further oxdiation was inhibited e.g. by addition of bromoethane sulfonate.

In conclusion, the following basic reactions were observed with aromatic compounds so far:

Decarboxylations, reductive dehydroxylations, reductive deaminations and reductive dehalogenations. The latter reaction appears to attack halogen substituents preferentially in the meta-position to an existing carboxylic function (ref. 38) and, behaving so, resembles the reductive dehydroxylation of 3-hydroxybenzoate (ref. 37). All these reductive elimination reactions may mainly represent means of disposing of excess electrons for the fermenting bacteria involved. It has to be discussed now to which extent the bacteria benefit from the various reactions mentioned.

PART 2: ENERGETICAL IMPLICATIONS OF ANAEROBIC TRANSFORMATIONS

Most of the transformations of organic matter mentioned above were studied in our laboratory with cultures obtained from anoxic sediment and sludge samples by enrichment with the respective substrate as sole energy source. This approach only focuses on such transformation reactions which provide enough energy to the organisms involved that they can multiply at the expense of substrate degradation. "Cometabolic" activities which do not contribute to the energy budget of the bacteria in play are necessarily overlooked with this method: therefore, failure of finding significant transformation of a certain compound should not be taken as an argument that its degradation is not possible in an anoxic environment; perhaps the substrate is transformed slowly by the resident bacterial population, but at least one of the reaction steps involved is catalyzed by a bacterium which does not benefit sufficiently from this activity and cannot be transferred to subcultures, therefore. This aspect is of basic importance in methanogenic transformations because the overall free energy change available is small (with glucose about 15% of that of aerobic oxidation) and has to be shared by two, three or even more different trophic groups of bacteria. For example carboxylation of a dicarboxylic acid releases about 17 kJ per mol (ref. 39). This amount of energy is small compared to the energy necessary for ATP synthesis (about 60 kJ per mol), and at least three decarboxylation reactions would be required for synthesis of one single ATP. A bacterium was recently isolated which grows by coupling a decarboxylation reaction with ATP synthesis via a sodium ion gradient across the cytoplasmic membrane (ref. 49). At the moment, the equivalent of one third of the energy required for ATP synthesis is the minimum quantum we can think of to allow

ATP synthesis and growth because three protons or other ions have to cross the membrane to synthesize one ATP by a membrane-bound ATPase (ref. 41).

Decarboxylation of benzoate derivatives to the respective phenols releases free energy in the range of 12-15 kJ per mol, equivalent to about one third of an ATP. So far, there is no indication of an energy conservation linked to this reaction; Growth yields with the carboxylated and the decarboxylated substrated did not differ significantly. Perhaps the decarboxylation energy can to some extent be conserved in ion gradients to drive substrate uptake.

The redox potential of couples of hydroxylated, aminated of halogenated aromatics with their respective reduced counterparts is in the range of about - 100 mV (refs. 34,37). Thus, electron transfer from alcohols to these electron acceptors would allow a small but significant energy conservation. If electrons at the potential of molecular hydrogen could be used, even electron transport phosphorylation with the concomitant synthesis of about one mol ATP per mol electrons transported could be possible. But perhaps the main role of these reductive elimination reactions is simply to release high-potential electrons which otherwise would require either to excrete reduced fermentation products instead of acetate and thus to waive a substrate linked phosphorylation, or to invest part of substrate-derived ATP into a reversed electron transport to allow hydrogen release (ref. 41). In the case of reductive dechlorination it has been demonstrated that the organism catalyzing this reaction is not involved any further in the degradation of the substrate molecule (ref. 42). Thus this bacterium has to obtain all its energy for growth from the reductive dehalogenation reaction and the source of the electrons used is still unknown. It remains to be examined to which extent the bacteria catalyzing reductive dehydroxylations and deaminations profit from these reactions. So far it is in most cases still open whether they also participate in the further substrate degradation sequence. Similar questions have to be asked with respect to the hydration of terminally unsaturated hydrocarbons mentioned above. Further work has to be done on the isolation of pure or defined mixed cultures from the existing enrichments.

The question to which extent the bacteria transforming organic compounds anaerobically profit from the reactions catalyzed also

bears some ecological and technological aspects. A microbial community involved in degradation of a certain substrate in a natural ecosystem can only adapt to quantitative and qualitative fluctuations in their diet composition if they all are positively influenced by the degradation process meaning if they all profit from the job they are doing. If one of the first steps in substrate transformation does not allow growth of the catalyzing organisms the whole reaction chain will operate continually at a low rate which cannot be raised by substrate feeding. It is needless to mention that such a system also could not be enriched for in e.g. anaerobic waste treatment setups.

CONCLUSIONS

The various types of anaerobic transformation reactions discovered in the recent past have broadened the range of substrates subject to anaerobic attack considerably, and the assumption is justified that we are only at the beginning of exploring the potentials and limits of anaerobic biodegradation. Since the type of primary attack on the substrate is basically different from that of oxygenase reactions (meaning: nucleophilic rather than electrophilic substitutions/additions) it is not astonishing that several substrates are degraded anaerobically even better than aerobically. Unfortunately, the bacteria involved in these transformations are only difficult to enrich because their energy yields and growth rates are extremely low. Nonetheless, the search for pure or defined cultures is worth the effort since only such cultures can help to understand the mutual relationship of bacteria in a microbial community.

This understanding is necessary to allow predictions on the fate of synthetic compounds, e.g. xenobiotics, in our natural environment to which also anoxic habitats contribute to a considerable degree, last but not least in our own intestinal system. We can no longer afford to neglect anaerobic transformations as being only of minor environmental importance.

Knowledge of th: principles and limits of anaerobic degradation is also necessary to design suited systems for anaerobic treatment of dissolved and solid waste materials. Since scientivic exploration on this field has just only started we can hope that most of the present energy-wasting aerobic sewage treatment plants can be replaced by anaerobic counterparts in the nearer future. The experience in this matter so far taught us that it is

more promising to look fbr metabolic capacities already exisiting in nature rather than constructing labile artificial abilities in genetically manipulated bacteria which have no chance to survive in a system more or less distantly related to natural conditions.

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